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HARPIN ADSORBED SILVER NANOPARTICLES INDUCED ANTI-OXIDANT ENZYMES IN TOBACCO

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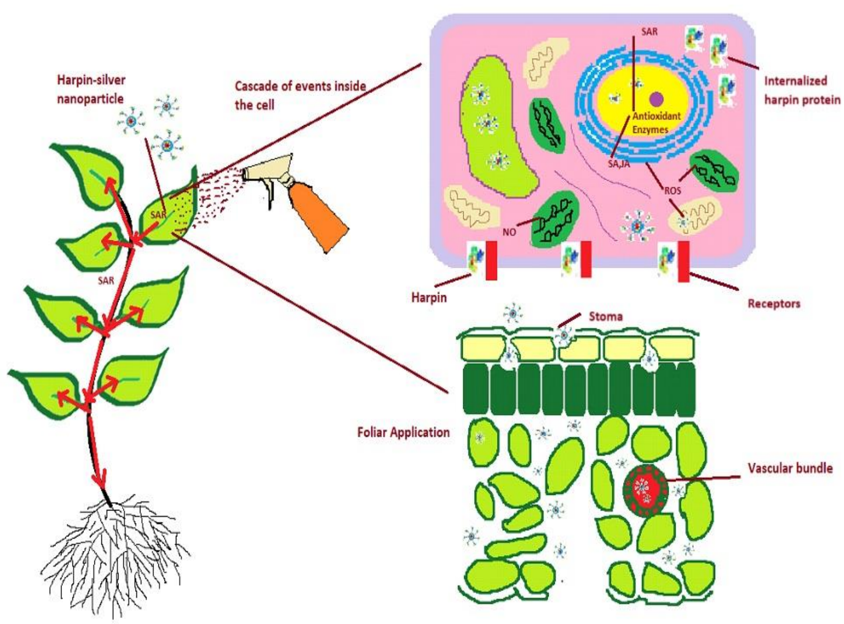
ABSTRACT

Elicitors such as harpin exhibit hypersensitive response upon infiltration in non-host plants. Foliar spray of harpin is not economical due to its poor bioavailability on the plant. Harpin in the form of nanoparticles would increase its appearance in the cellular environment. So here we tested harpin coated silver nanoparticles (H-AgNPs) as a foliar spray on tobacco plants. Reproducible, stable small sized (~ 44 nm) silver nanoparticles (AgNPs) were prepared by seed mediated method and adhered with harpin. Conjugation of harpin was confirmed by elemental analysis and UV-Visible spectrophotometry. Harpin attachment resulted in increased in size of H-AgNPs, determined by transmission electron microscopy and zeta sizer observations. Obtained results revealed incremental rise in ascorbate peroxidase, glutathione reductase and peroxidase within 12 hours. The outcome of the experiments showed that H-AgNPs with nanozyme nature could mitigate the oxidative stress in plants. It is clearly evident that H-AgNPs are internalized and pronounced elicitor activities when AgNPs and harpins are together . This method of application resulted in decreasing the effective concentration of the harpin in inducing micro hypersensitive response in the plant and proved that H-AgNPs can be used as nanoelicitors at appropriate concentrations.

Keywords: Harpin, Silver, Nanoparticles, Plants

GRAPHICAL ABSTRACT



INTRODUCTION

Non-host plants when invaded by pathogenic bacteria induce hypersensitive response (HR) by proteinaceous elicitors like harpins. They were initially recognized while discovering the bacterial factors involved in HR of fire blight causing *Erwinia amylovora*. The gene which encodes the harpin was identified as *hrpN* (1). Infiltration of the *hrpZ* (34.7 kDa) which is secreted by *P. syringae* pv. *Syringae* on tobacco plants elicits HR. Harpin was commercialized as a biopesticide under the trade name of messenger and known to activate the basal defense responses in plants but the poor assimilation into the microenvironment of plant cells hampering its usage as a topical spray. Repeated application of harpin is required to activate long lasting systemic acquired resistance (SAR) in plants against phytopathogens. Consistent size of harpin as a nanoparticle may be helpful in reaching its putative receptor hypothesized to be present in the plasma membrane or rigid cell wall of plant cells.

Nanoparticles (NPs) display a size range of within 100 nm (2). They differ in terms of possessing more surface area to volume ratio with their original matter. Owing to their minute size they can enter into various tissues and cells in plants and animals. NPs are utilized to carry different molecules like drugs, proteins, carbohydrates to the targeted locations as they can adsorb, bind and assimilate with them. The narrow dimensions of the NPs make them specialized carriers of the associated molecules in agriculture and medicine. Among those silver nanoparticles (AgNPs) are significant in the field of medicine in wound treatments due to their antifungal, anti-bacterial and non-toxic nature under considerable concentrations. Application of AgNPs in agriculture are scanty in comparison with medicine.

Various metal oxide nanoparticles are employed in precision agriculture. Pesticides made of AgNPs are utilized to increase the crop productivity and growth of the plant (3–6). Companies like Bayer, Syngenta and Monsanto are using Ag as a major ingredient in their agricultural products (7). Positive and negative plant physiological parameters observed with the treatment of silver. Application of AgNPs resulted in change in the biochemical, physiological and metabolic processes by changing the growth, nutrient and water uptake, ROS, oxidative stress, photosynthesis, transpiration and respiration (8–11). The induced growth rate in soybean by 15 nm AgNPs was evidenced by Mustafa et al. (12). Fruit yield and cucumber weight drastically improved after application with 500 ppm AgNPs (13). Adverse effects were reported on the activity of anti-oxidant enzymes by the nanoparticles (14–16). Agriculture is variously influenced by the effect of nanoparticles by their presence and persistence as they are becoming inevitable (17).

Nanoparticles are aided in the targeted delivery which increases their absorption rate and lowers the required biological entity to induce HR. Foliar application of harpin in the form of nanoparticles proved to be effective and increases the chances of absorption through stomata (18–20). Parameters such as size, morphology, surface coating, composition and concentration would prominently affect the physiological aspects of a plant which can be determined by advanced molecular approaches like proteomics and genomics. Evidence reported that AuNPs coated with harpin upregulated the PR proteins by intra and intercellular localization (Kongala et al., 2021). Harpin encapsulated chitosan nanoparticles resulted in upregulation of defense enzymes upon foliar application (18,20). With the increasing evidence of AgNPs utilization in agriculture due to their nanozyme nature, we studied the effect of harpin adsorbed AgNPs as foliar spray on tobacco plants. This is the maiden approach in which Ag conjugated with harpin in the form of NP which could be a promising tool as a nanoelicitor at the given concentrations without harming the environment.

MATERIAL AND METODS

Synthesis of Colloidal AgNPs

The AgNPs used in this study are synthesized by seed method (21). Initially seed solution is prepared by quickly mixing 500 µL of 100mM $NaBH_4$ into an aqueous solution having 500 µL of 10 mM silver nitrate and 20 ml of 0.001 M sodium citrate under constant stirring for 5 min and incubated for 90 min. 3000 µL of silver seed solution and an aqueous solution of sodium citrate (1 mM) is added to boiled 100 ml of $AgNO_3$ solution containing 1mM concentration. The solution turns to greenish red upon heating and allowed to cool to room temperature, centrifugation done at 8000 rpm at room temperature for 10 min and purified. The pellet is washed three times to remove the excess silver ions with deionized water. Biofunctionalization is done by adding 100 µg of harpin protein (approximately 100 µl) to each 1 ml of AgNPs drop by drop with gentle mixing for 120 min at 30 ± 2^0 C results in the formation of harpin adsorbed silver nanoparticles (H- AgNPs).

Physicochemical characterization of AgNPs and H-AgNPs

Nanoparticle size is determined by transmission electron microscopy (TEM) which is operated at low voltage. Small aliquots of Protein coated and uncoated AgNPs are taken onto copper grids which were coated with carbon, dried and observed. The adsorption of proteins on the nanoparticle surface is analysed by UV-Visible spectrophotometer (Shimadzu UV-vis 1800) at 200-600 nm and recorded by uniprobe software. Energy dispersive X-ray analysis is carried out by keeping 2-3 µL of NP solution on the stub at 20kV with Field Emission Scanning Electron Microscope (model Ultra 55, Carl Zeiss). The size and zeta potential of AgNPs and harpin adsorbed AgNPs measured by Zeta sizer (Malvern, Netherlands).

Study of defense responses in the tobacco plant

Tobacco cultivar *(Nicotiana tabacum* cv. *Xanthi)* was maintained in a glasshouse with required conditions (19). 5-6 broadly expanded tobacco leaves were treated with 1000 µL of AgNPs, H- AgNPs, harpin (100 µg/mL) and water. Biological triplicates of leaf samples are collected in liquid nitrogen for further analysis. The remaining treatments are compared with water which serves as control.

Defense enzyme activities

One gram of leaf tissue is macerated in 1 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.5) having 8% (w/v) polyvinylpyrrolidone, 1 mM phenylmethylsulphonyl fluoride,1 mM polyethylene glycol and 0.01% (v/v) Triton X-100. After homogenization centrifugation is carried out at 16000 g for 20 min at 4^0C and the upper aqueous phase is utilized for determining enzymatic activities. Peroxidase (POD) activity is measured by following Rani et al., (22). The enzymatic activity of AsPOD (Ascorbate peroxidase) (EC 1.11.1.11) was determined according to Mano et al., (23). Hasanuzzaman method is used for determining GR (Glutathione reductase) (EC 1.6.4.2) activity (24). All the enzyme activities are expressed as µmol/gram on a fresh weight basis.

Statistical analysis

All data presented are the mean ± SD of three separate experiments conducted in triplicate. The standard error of the mean was represented by error bars.

RESULTS AND DISCUSSION

Harpins are bacterial proteins which elicit HR in plants. Topical application of alone harpin is not rendering resistance as most of it is undergoing degradation. Inter and intracellular delivery of this bacterial elicitor known to cause SAR in plants (25,26). Harpin in the form of nanoparticles may have the chance of increasing its bioavailability after entering through the epidermal openings. Movement of nanoparticles depends upon the size as it is the main constraint for long distance transport. Nanoparticles with proper size (40, 50 nm) can move inside the plant cells (26–28). The stomatal pores (100 nm) are the easy paths through which to get into the plant environment (29). In this work we reported harpin (100 µg/mL) attached to Ag nanoparticles (~ 44 nm) as a foliar spray in tobacco plants.

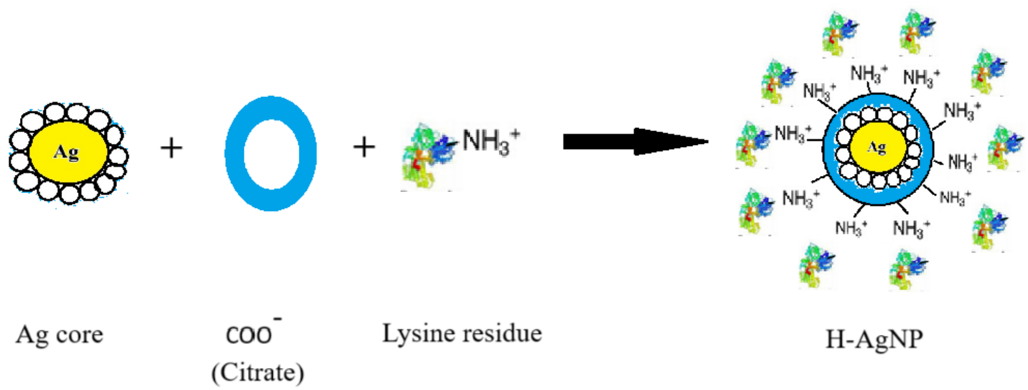


Figure 1. Illustration of formation of harpin conjugated silver nanoparticles (H-AgNPs). Schematic representation showing citrate reduced silver nanoparticle. Negatively charged acetate surrounding the Ag, in citrate reduction, conjugated with positively charged lysine residues of the harpin protein.

In the citrate reduction of AgNPs , monodisperse nanoparticles are formed . The AgNPs are capped with citrate ions which renders negative charge shows affinity towards positively charged biomolecules like lysine containing proteins (30) (Figure 1). AgNPs are stabilized by surface functionalization which improves intracellular stability besides keeping the characteristic features of nanoparticles. Biofunctionalization of AgNPs with biomolecules alters the morphology and composition of the surface of nanoparticles (31). Surface functionalization gives the advantage of aqueous stability and enhances the attachment of biomolecules like peptides, antibodies, nucleic acids and amino acids which makes them further useful in clinical applications (32). The surface functional groups on the AgNPs and the charge exploit the association of biomolecules with the nanoparticles. Electrostatic stabilization induced by various capping agents make them to be used as delivery vehicles of drugs and aids in the controlled release of targeted drugs to the vicinity inside the cells which improves bioavailability of the released drug. The nature of adhered agents on the surface of AgNPs causes various changes in the biological and physical properties for their implication in various areas. The citrate ions not only imparts surface stabilization but also adds negative charge to AgNPs. The hydrophilic nature of citrate ions facilitates conjugation of biomolecules on the surface of AgNPs. Positively charged cationic peptides comprise amino acid residues like arginine and lysine when entangled to AgNPs facilitates easy transit into the cellular environment (33) and thereby subcellular compartments. The conjugation with cell penetrating peptides reduces the negative charge of AgNPs coated with citrate ions, further breaches the electrostatic barrier of cell membrane by diminishing negative charge and increases cellular internalization. The harpin molecule which contains 14 lysine residues electrostatically attaches to the surface of citrate coated AgNPs with their positively charged amine group. The bond formation between the carboxylic moiety of the citrate group and the NH_2 of lysine renders attachment of harpin on the surface of AgNPs (Figure 1). This positively charged lysine residue not only helps in the bioconjugation but also transports the AgNPs into the microenvironment of the plant cells. This is clearly evident by the induction of various defense enzymes such as Glutathione reductase, peroxidase, Ascorbate peroxidase and increase in the protein content in the plant cells.

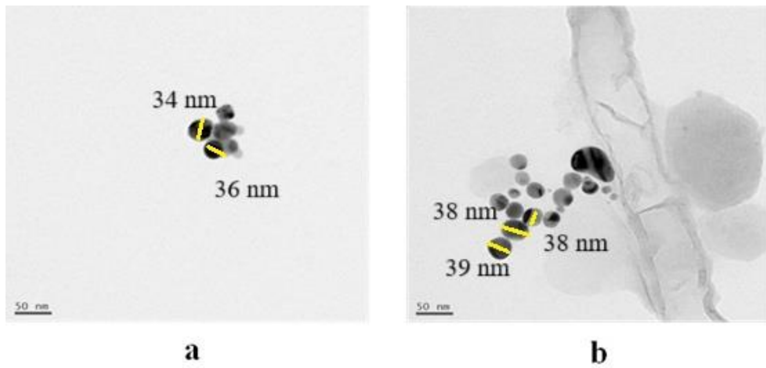


Figure 2. Characterization of silver nanoparticles with transmission electron micrographs (TEM). The morphology and size determination with transmission electron microscopy was done by placing an aliquot of sample on the carbon coated copper grid and allowed to air dry at room temperature. a) represents nascent AgNPs and b) is H-AgNPs. The sizes of the nanoparticles were ~ 35 and ~ 38 respectively

The TEM observations revealed the shape and morphology of the NPs. The AgNPs appear to be spherical and uniformly distributed (Figure 2). The bare AgNPs possess a size of ~ 35 nm and after harpin attachment the size increased to ~ 38 nm, indicating the successful conjugation. Elemental analysis of AgNPs and H-AgNPs clearly evidenced the presence of harpin on the corona of the AgNPs. In AgNPs the Ag was 12.88 atom % which was decreased to 1.39 in H-AgNPs. The oxygen amount in H-AgNPs considerably hiked to 62.53 atom % from 48.91 atom % in AgNPs. The carbon content decreased from 19.83 atom % in AgNPs to 3.38 atom % in H-AgNPs (Figure 4). The decrease of Ag and carbon content in H-AgNPs and increase in oxygen prominently describes the harpin adsorption. UV-Visible spectroscopy is widely employed in the structural characterization of AgNPs. Earlier reports showed an absorption spectrum at ~ 420 nm (21). The current work portrayed similar results. Naked AgNPs indicated a peak at ~ 407 nm whereas conjugated AgNPs showed a prominent peak at ~ 411 nm for H-AgNPs and ~ 414 nm with (Bovine serum albumin) BSA conjugated silver nanoparticles (B-AgNPs) (Figure 5). As BSA contains more lysine residues, it was also conjugated to AgNPs to prove that lysine residues facilitate attachment onto the negatively charged NP surface. The absorption peak shifted from 407 nm to 411 nm and 413 nm respectively for harpin and BSA coated AgNPs. These changes in the absorption peak renders the successful conjugation of the proteins on the surface of AgNPs. The hydrodynamic size of the bare AgNPs and harpin attached AgNPs was 41 and 44 nm and the zeta potential measurements showed -33.3 mV and -18.8 mV (Figure 3). The change in the zeta potential gives an indication of successful attachment of protein on the corona of nanoparticles.

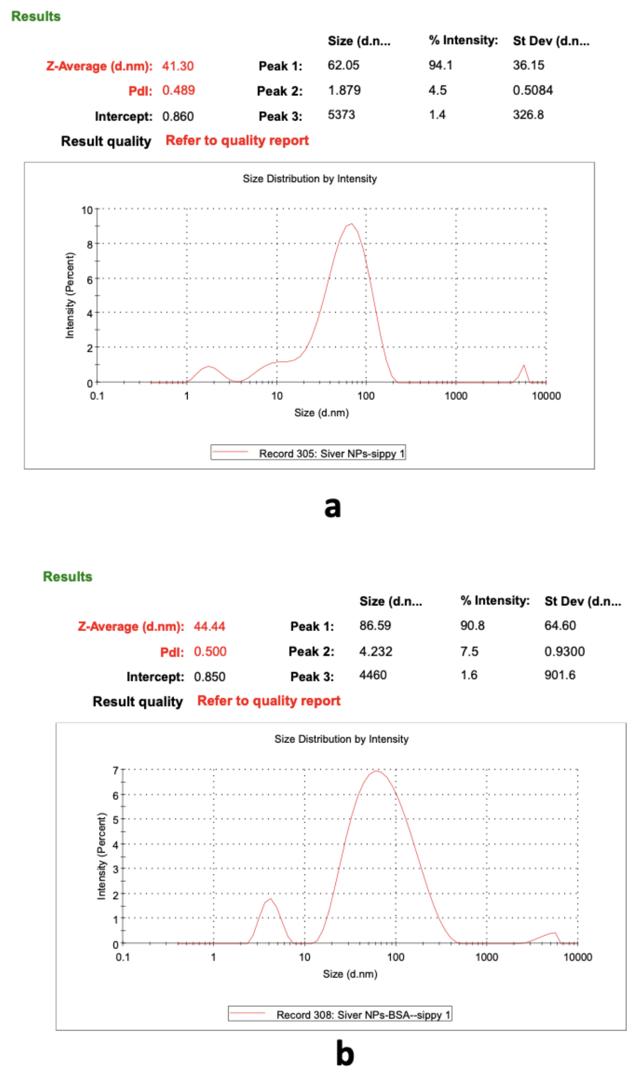
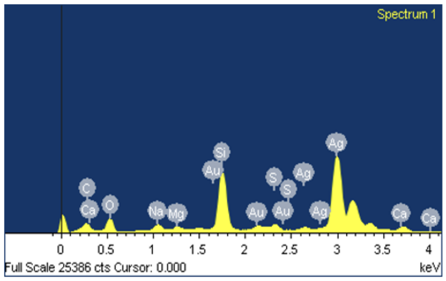


Figure 3. Results of hydrodynamic size. Hydrodynamic size of a) AgNPs and b) H- AgNPs. The bare AgNPs possess a size of 41 nm and harpin attached AgNPs was 44 nm indicating the adsorption of protein on the corona of nanoparticles.

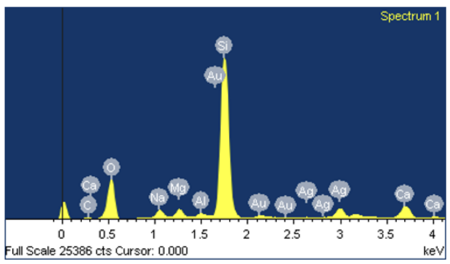
Biotic and abiotic stressors are the major reasons for the plant diseases (34), which are the impediments of agricultural productivity. Higher agricultural productivity is obtained by ceasing various environmental stresses to fulfil the ever growing population. ROS results from biotic and abiotic stress components and can be alleviated with the appropriate concentrations of anti-oxidant enzymes in plant defense responses. The plant's anti-oxidative defense system includes enzymes such as catalase, peroxidase, superoxide dismutase and enzymes of ascorbate- glutathione cycle (35). Anti- oxidant and oxidative damage of metallic nanoparticles relies on type, dosage, mode of exposure and modification of nanomaterials. Metallic oxide nanoparticles known to scavenge the free radicals by mixed valence states (36,37). They exhibit ROS scavenging activities and are known to have advantage (38–40). Chloroplast was protected by dextran stabilized CeO NPs at a concentration of 5 µM from ROS (41). Photosynthetic activity was enhanced in *Arabidopsis thaliana* by decreasing ROS under abiotic stress upon leaf infiltration with 0.05 g L^{-1} poly acrylic acid containing cesium oxide NPs (40,42). Djanaguiraman et al. observed that topical application of cesium oxide NPs 10 mg L^{-1} encountered oxidative stress in sorghum against drought (43). A meagre level of ROS levels were maintained by 2 mg L^{-1} of γ Fe_2O_3 NPs in *Brassica napus* under drought and improved resistance towards it (44). When 1 mg of Mn_3O_4 sprayed foliarly per plant, said to alleviate the oxidative stress and increased intracellular defense anti-oxidant enzymes in cucumber (38).

a



Elements	Weight %	Atomic %
C K	8.13	19.83
O K	26.72	48.91
Ag L	47.45	12.88

b



Elements	Weight %	Atomic %
C K	1.91	3.38
O K	47.22	62.53
Ag L	7.06	1.39

Figure 4. Elemental analysis of AgNPs and H-AgNPs. EDS profile of a) AgNPs and b) denotes H-AgNPs . Biofunctionalized (H-AgNPs) and nascent AgNPs showed prominent signals for Ag, oxygen and carbon. The profile of oxygen increased in H-AgNPs where as carbon and Ag content minimized. AgNPs exhibited a prominent signal for Ag and carbon than the H-AgNPs and decreased oxygen confirms the successful conjugation of harpin on Ag surface (H-AgNPs).

Plant cells are enrouted via anti-oxidative defense enzymes to combat oxidative damage caused by ROS for protection (45). The exposure to Ag and AgNPs significantly increased the antioxidant responses and upregulation of defense genes peroxidase, phenylalanine ammonia lyase, ethylene inducing xylanase besides improving hydrogen peroxide and malondialdehyde when compared to control (46). Increased ROS production and antioxidant defense enzymatic activities of peroxidase (POD), superoxide dismutase (SOD) were promoted finally caused an increase in phenol acids after exposure to AgNPs in *Ricinus* (47). Defense enzymes particularly superoxide dismutase (SOD) increased by 5.7 % on average, CAT by 17.3 %, POD by 7.9 %, Glutathione peroxidase (GPX) by 32.2 % and no significant impact by As POD even though it is having more affinity towards hydrogen peroxide, might be due to invariable H_2O_2 content (45). Similar results were observed in the current study. The anti-oxidant enzymatic activities increased with time.

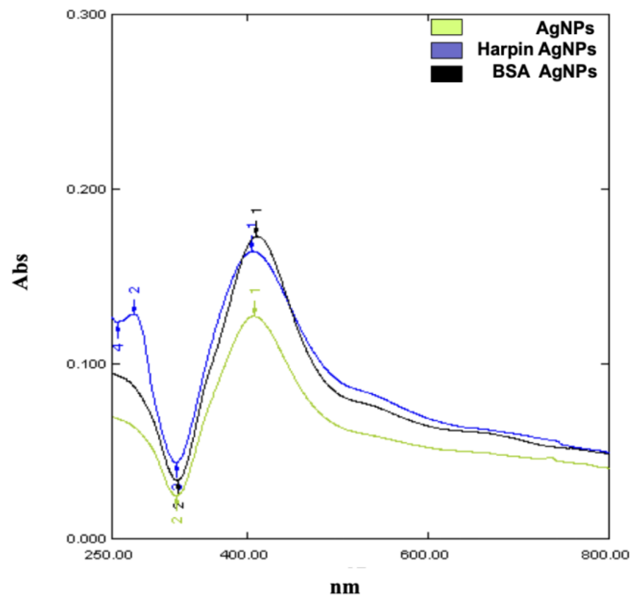


Figure 5. UV- visible spectroscopy observation of silver and its conjugates. UV- Visible spectroscopic analysis showed a peak at 407 nm for pure silver nanoparticles (Green line) and 411 nm peak for harpin adsorbed silver nanoparticles (H-AgNPs) (Blue line), 414 nm peak for Bovine serum albumin coated silver nanoparticles (B-AgNPs) (black line). A shift in the peak for both H-AgNPs and B-AgNPs confirms the presence of harpin on the Ag surface.

Peroxidase, Glutathione reductase, Ascorbate peroxidase activities were increased within 12 h. The POD activity gradually increased from the first hour and reached maximum extent by 3 h. In the first hour AgNPs treatment showed 3 folds and 4 folds in H-AgNPs treatment than the control plants. By 3 h the activity of POD reached maximum in H-AgNPs treatment which is almost 3.5 folds higher than the control and 2 folds in AgNPs. The POD activity gradually decreased from 3 h to 12 h. Quick accumulation of POD was observed in 3 h may be attributed to the harpin coating on the AgNPs surface (Figure 6a) . High amounts of POD, As POD and Catalase (CAT) directly indicates less ROS formation which is less toxic to the plants (48). It should be noted that the enzymatic activities gradually increased in case of POD up to 10^{th} day whereas As POD was increased up to 8 th day and then decreased when it reached to 10 th day. Observations concluded that POD accumulation was always higher in the H-AgNPs treatment than the AgNPs, clearly exhibiting the cumulative role of both AgNPs and harpin. The As POD activity was always higher than the control in H-AgNPs treatment . It is 1.6 and 1.5 folds higher than the control in the 1 and 3 h and lowered 0.8 folds than the control in 6 h and reached maximum activity which is doubled than the control in the 9 h and decreased after12 h (Figure 6b). The decrease in As POD and GR might be due to the generation of high levels of ROS and possible role of these enzymes in nullifying it. Regain of these enzymatic activities after ceasing the ROS was observed. The As POD activity by the AgNPs is always lower than the control except in the 9 h. But in H- AgNPs treatment its activity was higher than the control showing the prominent role of inducing defense enzymes by harpin. GR activity was 1.4, 1.6, 1.6, 1.8 and 1.6 folds increased than the control during 1, 3, 6, 9 and 12 h. The GR activity was slightly hiked than the control in 1 and 6 h and lowered in the remaining time intervals with AgNPs (Figure 6c). GR expression was upregulated in *A. thaliana* upon treatment with AgNPs when compared to silver ions, depending upon the size and concentration across species (8). The level of GR expression abruptly increased after harpin was conjugated to the AgNPs indicating the elicitor role of harpin. The increase in anti-oxidant enzymes in the cells opining that AgNPs attached with harpin under considerable concentrations will have good implications in alleviating stress in tobacco plants.

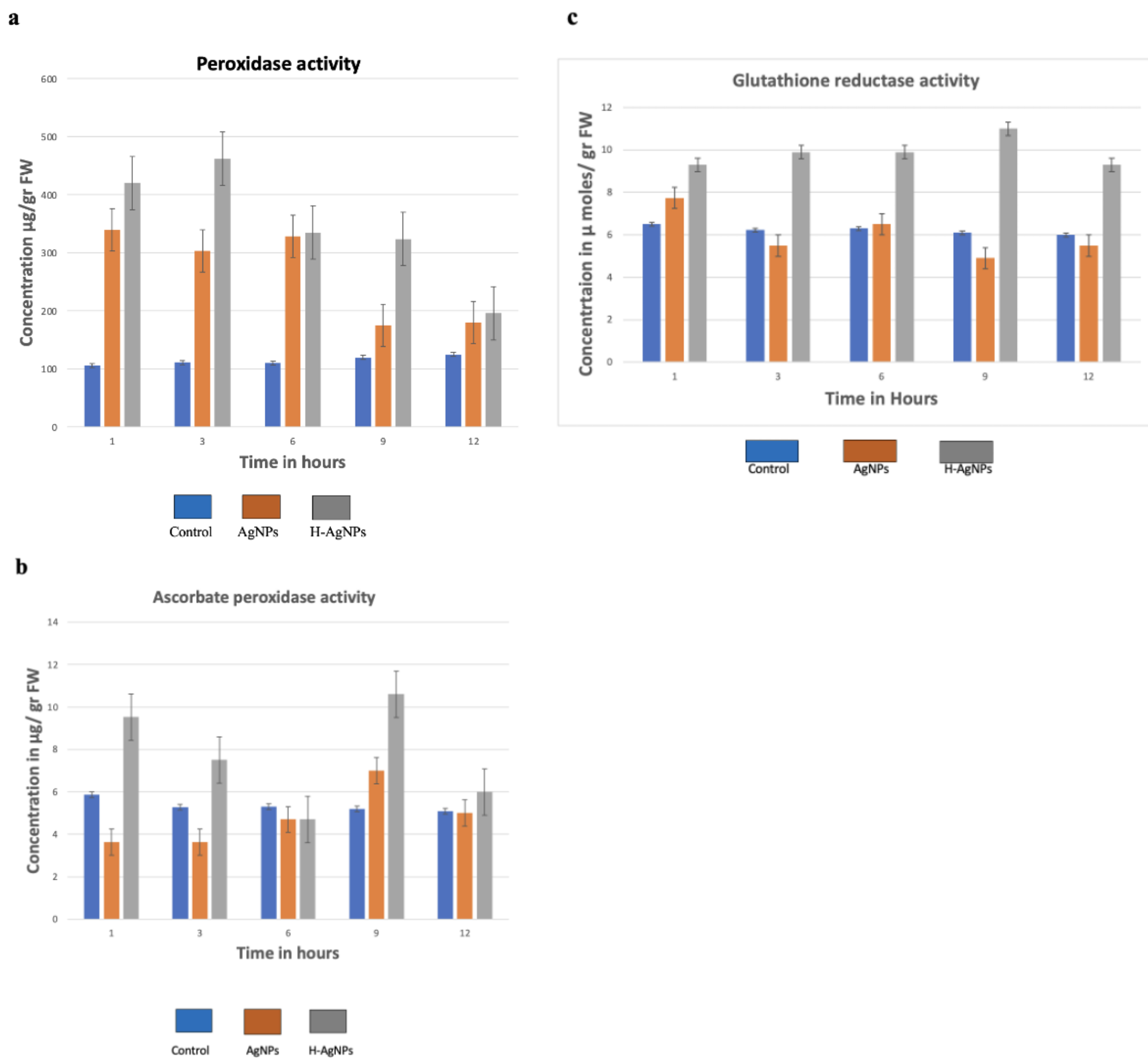


Figure 6. Expression of defense enzymes upon treatment with nanoparticles. Increased activities of a) Peroxidase, b) Ascorbate peroxidase and c) Glutathione reductase, in tobacco with Control, AgNPs and H-AgNPs after 1 , 3 , 6 , 9 , 12 h. Enzyme activities were expressed in µmol/gr FW. Error bars indicate standard error from three different experiments (mean ± SD, n=3).

CONCLUSION

Harpin was successfully conjugated to AgNPs and confirmed with energy dispersive X- ray analysis UV-Visible spectrophotometry and Zeta sizer. The foliar spray of H-AgNPs in tobacco markedly increased the defense anti-oxidant enzymes within 12 h. In view of replacing the inherent anti-oxidant enzymes to mitigate ROS by H-AgNPs, they are considered to be a promising tool to boost plants' innate immunity against various plant pathogens and during stress conditions. Therefore we believe that the nanoelicitor nature of H-AgNPs marked as a strategy to improve the resistance in the physiological aspects of the plants during stress.

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CONFLICT OF INTERESTS

The author declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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